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14. ABSTRACT

The etiologic agent of plague is the Gram negative bacterium Yersinia pestis. Y. pestis is a concern as one of the microorganisms with potential for use against civilian or military populations as a biological warfare/ biological terrorism agent. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. The studies reported herein explore the ability of a novel adjuvant, designated LT(R192G), to promote the rapid development of long-lasting, high titer antibodies against a recombinant plague antigen (F1-V) and protection in a murine model. Subsequent studies will be performed in non-human primates. Different routes of administration are examined to test the hypothesis that heterologous boosting will be more effective than homologous boosting at increasing the magnitude and/or duration of the antibody response.

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INTRODUCTION

The etiologic agent of plague is the Gram-negative bacterium *Yersinia pestis*. *Y. pestis* is a concern as one of the microorganisms with potential for use against civilian or military populations as an agent of biological warfare or biological terrorism. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. A number of potential subunit vaccine against plague have been evaluated for immunogenicity and protective efficacy. The two most promising are the *Y. pestis* proteins F1 and V. F1 is a capsular protein located on the surface of the bacterium and the V-antigen is a component of the Type III secretion system. In previous studies, combined immunization with native F1 and recombinant V (rV) in a two-dose regimen afforded full protection in mice against subcutaneous challenge with *Y. pestis* (15) and the anti-F1 and anti-V titers, especially of the IgG1 sub-class, correlated significantly with protection in BALB/c mice. Male and female CBA, C57/BL6 and CB6F1 mice were also protected against injected and aerosol challenge with *Y. pestis* following immunization with two doses of rF1 and rV (6). The combination or fusion of F1 and V has been has an additive protective effect in the murine model when compared to either antigen alone (3-5, 11, 13, 14). Heath et al. (5) reported construction of a an F1-V fusion consisting of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V-antigen. F1-V was shown to provide excellent protection against both subcutaneous and aerosol challenge and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild type $F1^+$ *Y. pestis* or to naturally occurring $F1^-$ variants.

Soluble protein-based vaccines, such as F1-V, are generally administered subcutaneously or intramuscularly in the presence of an aluminum salt adjuvant. For most proteins, this is an effective means of inducing serum antibody against the antigen (i.e., tetanus and diphtheria toxoid). Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal, oral, rectal) and transcutaneous immunization in the presence of an appropriate adjuvant have been shown to induce humoral and cellular immune response in both the systemic and mucosal compartments. Alternating routes for delivery of the priming dose and booster dose in immunizations, so called “prime-boost” strategies have also been examined for the ability to induce high-titer, long-lasting humoral responses and have the potential direct or redirect the immune response to one compartment or another. This may be particularly useful for development of vaccines against agents that may be delivered by aerosol, where the respiratory mucosa would be the first point of productive contact between the organism and the host.

In the current contract, we examined different prime/boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examine the effect of different prime/boost regimes on the compartmentalization of the ensuing immune response. For parenteral immunization, F1-V is adsorbed to aluminum hydroxide, which is commonly used as an adjuvant for parenterally administered vaccines. Mucosally and transcutaneously administered vaccines are usually not immunogenic and also require the presence of an appropriate adjuvant. In the current studies, we utilize a mutant of the heat-labile enterotoxin of *Escherichia coli*, designated LT(R192G), that has been shown to be effective when administered mucosally (orally, rectally, intranasally) or transcutaneously in a variety of animal models and in humans.

BODY

This project is organized into two Specific Aims that constitute the Technical Objectives of the proposal.

Specific Aim 1. Optimize the Immune Response to F1-V in a Murine Model. In the first specific aim, we examine the ability of LT(R192G) to function as an adjuvant for F1-V when delivered mucosally or transcutaneously and the ability of adjuvanted mucosal or transcutaneous immunization to serve as a booster for parenteral priming. The primary objective of this aim is to optimize immunization to achieve a rapid anti-F1-V antibody response of high titer and of long duration. Another objective of this aim is to determine if the antibody response to both antigens, F1 and V, is sustained. Aerosol challenge of immunized mice will be conducted to correlate the induction of serum and mucosal antibodies with protection. The optimum prime/boost regimen from these studies, as defined by antibody responses and confirmed by challenge, will subsequently be examined in Non-Human Primates (NHP).

Status of Specific Aim 1. All of the requirement of this aim were completed at the time of the last report filing. The findings from these studies have been published in two manuscripts (see below) and the data were used to down-select candidate vaccine approaches for Specific Aim 2.

Specific Aim 2 (Revised). Evaluate the Immune Response to F1-V in Non-Human Primates. The objective of this aim is to test the comparative efficacy of USAMRIID's current F1-V plague vaccine candidate with or without mucosal adjuvant administered in heterologous vaccination schemes against aerosol challenge in nonhuman primates. The candidate heterologous vaccination approaches will be down-selected from the mouse model of plague (Specific Aim 1) prior to testing in NHP. We hypothesize that heterologous prime/boost with rF1-V in conjunction with the mucosal adjuvant LT(R192G) or Alhydrogel will be superior to homologous route vaccination with rF1-V in Alhydrogel.

Status of Specific Aim 2. The original Scope of Work and timetable projected completion of Specific Aim 1 by the end of the first calendar year and completion of Specific Aim 2 by the end of the second calendar year. A number of obstacles prevented the timely initiation of Specific Aim 2. The first was a source of antigen, F1-V. There have been increased demands on limited supplies and we were not always able to obtain sufficient quantities of this antigen from USAMRIID in a timely manner. One provision of the contract was that USAMRIID had the option of requiring a mouse challenge study before proceeding to the NHP studies. At USAMRIID's request, animals to be challenged were immunized at Tulane University and shipped to USAMRIID for challenge. There was some delay while the composition of the challenge groups was agreed upon and an additional delay waiting for a window of opportunity to open at USAMRIID. A no-cost extension was granted to allow these studies to continue. Those studies were completed and reported in the last progress report. Subsequent to that mouse challenge study, USAMRIID investigators indicated a desire to expand the number of animals in the NHP study from the budgeted 8 to a total or 36, employ telemetry (requiring surgical manipulation of each animal), and charge the contract for the additional animals, telemetry devices, surgery, and challenge (not part of the original contract). The original budget was based on 8 animals to be immunized at the Tulane National Primate Research Center and shipped to USAMRIID where challenge would be conducted for no charge, so there was simply no way to cover the additional costs within the existing budget. Consequently, a decision was made to move that portion of the study to USAMRIID and to use the balance of the funds at Tulane ($120,875) to reimburse USAMRIID to partially offset the cost of the expanded study. A CRDA (USAMRIID Control No. W81XWH-06-0270) formalizing the revised study plan and reimbursement was instituted and became effective on July 7, 2006 (ending date July 7, 2008). Those studies

An additional delay occurred due to the disruption of operations associated with Hurricane Katrina. All

operations at Tulane University were effectively shut-down between August 29, 2005 and January 1, 2006.

KEY RESEARCH ACCOMPLISHMENTS AT TULANE

- Intranasal and subcutaneous immunization are essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route.
- Heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses.
- In no case was heterologous boosting inferior to homologous boosting and in three specific cases heterologous boosting was more effective than homologous boosting.
- IN and SC priming were more effective than TCI priming for induction of serum anti-F1V IgG1 when the boost was administered by any route and not different from one another through six-months post-primary immunization.
- With respect to BAL responses, either IN or SC prime followed by any boosting route induced significantly higher BAL anti-F1-V IgG1 than TCI priming, at least through six-months post-primary immunization, clearly demonstrating that either IN or SC priming may be effective when a bronchioalveolar response is desired.
- As single SC immunization with F1-V alone, with or without alum as an adjuvant, was sufficient to protect mice against aerosol challenge with 70 LD50 of *Y. pestis* CO92.
- IN prime and boost with LT(R192G) as an adjuvant provided solid (100%) protection against aerosol challenge with 70 LD50 of *Y. pestis* CO92.

Experimental Summary of Heterologous Prime Boost Vaccination with rF1-V in African Green Monkeys (AGM) at USAMRIID.

There was considerable discussion between investigators at Tulane and USAMRIID regarding the appropriate species of NHP to use for challenge studies. On the one hand, AGMs are a reasonable model for pulmonary plague but notoriously difficult to protect against aerosol challenge by SC immunization, with even multiple doses containing relatively large amounts of F1-V. *Cynomolgus* macaques, on the other hand, are not difficult to protect against aerosol challenge by SC immunization with F1-V. The question then was whether to try and convert a non-protective antigen to a protective antigen in AGMs by addition of the novel adjuvant LT(R192G) and heterologous prime - boost or, alternatively, to evaluate protection of *Cynomolgus* macaques by alternating the route of prime and boost. A decision was made to pursue the studies in AGMs.

<u>NHP Experimental Design and General procedures</u>

This experiment was designed to determine the efficacy of F1-V administered via a number of homologous and heterologous routes in combination with aluminum hydroxide or LT(R192G) in protecting AGMs against a lethal aerosol challenge with virulent *Y. pestis*. The routes and doses were down selected from the rodent studies previously reported.

Vaccine groups[a]	n	Prime		Boost	
		Antigen (μg)	Adjuvant (μg)	Antigen (μg)	Adjuvant (μg)
SCa x INr	6	150	850 Al	150	50 LT
INr x SCa	6	150	50 LT	150	850 Al
SCa x SCa	2	150	850 Al	150	850 Al
IN x SC	2	NA	50 LT	NA	850 Al

[a]For parenteral immunization, 0.5 ml F1-V was adsorbed to aluminum hydroxide (SCa) or the placebo was given alone (SC). For mucosal immunizations, 0.5 ml was administered (250 μl/nares). The F1-V was admixed with LT(R192G) (INr) or LT(R192G) was administered alone (IN).

USAMRIID IACUC protocol A05-08 describes in detail all of the procedures used for the animal experiments. All animals were examined at least daily pre-exposure and twice each day post-exposure. Telemetry data were recorded on each animal from the time of exposure. A full necropsy was performed to include collection of frozen and fixed tissues on all non-survivors.

Sixteen AGMs were divided into two groups of six vaccinates each and two control groups of two each. The vaccinates were vaccinated on day 0 and 36 with 150 μg of rF1-V combined with Alhydrogel or LT(R192G) by the specified routes while the group of 2 controls received traditional homologous rF1-V in Alhydrogel by the SC route or placebo (adjuvant only).

Eight weeks after the second dose of vaccine, NHPs were challenged by aerosol with approximately 50-200 LD50 of *Y. pestis* strain CO92 using a head-only dynamic aerosol system within a class III safety cabinet.

Only two of the 14 vaccinates survived challenge, both in the SCa x INr group. Unfortunately, the difference in survival between treated and control groups is not statistically significant. Preliminary data were provided on serum and BAL antibody and measurement of antigen recall responses, but further analysis is required.

Although this is the final report on this project, USAMRIID retains the balance of the unexpended funds and can pursue additional vaccine groups, routes, adjuvants, or different species of NHP.

REPORTABLE OUTCOMES

Glynn A, Freytag LC, Clements JD. 2005. Effect of homologous and heterologous prime-boost on the immune response to recombinant plague antigens. Vaccine 23:1957-1965.

Glynn, A., C. J. Roy, B. S. Powell, J. J. Adamovicz, L. C. Freytag, and J. D. Clements. 2005. Protection against aerosolized *Yersinia pestis* challenge following homologous and heterologous prime-boost with recombinant plague antigens. Infect. Immun. 73:5256-5261.

CONCLUSIONS

In the rodent studies, we examined different prime - boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examined the effect of different prime -boost regimes on the compartmentalization of the ensuing immune response.

The most significant findings of the immunization study are that 1) IN and SC immunizations are both effective and essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route, 2) heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses, and 3) anti-F1 and anti-V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. As with previously published studies, there were still detectable levels of circulating anti-F1-V antibodies even one year post-primary immunization.

The observation that heterologous boosting may, in some cases, produce higher and more sustained antibody responses than homologous boosting is consistent with other reports that have examined this question (1, 7-10, 12). Most recently, Lauterslager et al. (7) demonstrated that oral (PO) boosting with ovalbumin was more effective in animals primed IN, SC, or intraperitoneally than PO (homologous) boosting with the same antigen. Similarly, Nicholas et al.
(9) demonstrated that SC immunization was effective for priming animals subsequently boosted SC or IN (but not PO) with chimeric virus particles expressing a 17-mer peptide sequence from canine parvovirus (CPMV). In those studies, animals primed IN and boosted SC developed significantly higher serum anti-CPMV IgG2a responses than did animals primed IN and boosted IN. In the study of Baca-Estrada et al. (1) mice immunized SC with formalin killed whole cells and then boosted IN with formalin killed whole cells, either alone or formulated in liposomes, developed higher serum and BAL anti-*Y. pestis* antibody and higher systemic cell-mediated immune responses than did animals boosted SC. Our findings are in agreement with these studies, all of which demonstrate that heterologous boosting can be as or more effective that homologous boosting for induction of serum antibodies. Since serum IgG1 has been shown to be protective against aerosolized *Y. pestis*, mucosal IgA was not examined in the current study. However, Baca-Estrada et al. (1), Lauterslager et al. (7), and Nicholas et al. (9) each demonstrated that heterologous boosting could also induce significant mucosal IgA responses.

The fact that IN and TC boosting of SC-primed animals generated higher levels of anti-F1-V antibodies than homologous SC boosting is interesting and could be explained by the distribution of T effector-memory cells to the peripheral tissues following SC priming where they would be available to interact with cognate antigen applied mucosally or transcutaneously in the context of an appropriate adjuvant (e.g., LT(R192G)) (2). However, SC boosting of IN and TC primed animals was also more effective than homologous IN or TC boosting for induction of serum atiF1-V antibodies, suggesting that a more global immunological phenomenon may be functioning here. Moreover, the adjuvant employed for IN and TC immunizations may also play a role. A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells as well as interacting directly with T-helper cells, B-cells, and epithelial cells. Both the Lauterslager et al. (7) and Nicholas et al. (9) utilized cholera toxin as a mucosal adjuvant. Clearly, the role of the adjuvant in controlling these outcomes requires further investigation.

The amount of antigen delivered by each route may also contribute to the outcome. Thus, the observation that both IN and SC regimens induced significantly higher levels of serum and BAL anti-F1-V IgG1 than did TC immunization with the same antigen may be influenced by the amount of antigen delivered. It is possible that the responses to TC prime and boost would be higher if larger amounts of antigen were applied or other methods were used to make uptake of transcutaneously administered antigens more efficient.

In the NHP studies, we observed protection only in two of six animals primed SC and boosted IN with rF1-V in combination with alum or LT(R192G), respectively. None of the other groups were protected and there were no statistically significant differences in survival or mean time to death. It is possible that AGMs cannot be

protected by rF1-V without heroic measures (i.e., multiple high doses of antigen). Unfortunately, we do not know if humans are more similar to the difficult to protect AGMs or the more easily protected *Cynomolgus* macaques. Future studies could focus on the relative differences in immune responses by AGMs, Cynos, and humans to rF1-V or could combine F1-V with a different antigen known to elicit protection in AGMs (i.e., tetanus toxoid).

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Effect of homologous and heterologous prime–boost on the immune response to recombinant plague antigens

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Abstract

Among the pathogens that have been identified as potential agents of biological warfare or bioterrorism, *Yersinia pestis* is one of the main concerns due to the severity and potential transmissibility of the pneumonic form of the disease in humans. There are no approved vaccines for protection against pneumonic plague, but a *Y. pestis*-derived fusion protein (F1–V) has shown great promise as a protective antigen in murine studies. In the current study, we examine different prime–boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of recombinant F1–V to promote the development of long-lasting, high-titer antibodies. The most significant findings of the study reported here are that (1) intranasal and subcutaneous immunizations are both effective and essentially equivalent for induction of serum and bronchioalveolar anti-F1–V IgG1 responses when a single booster dose is administered by the same (homologous) route, (2) heterologous boosting can be as or more effective than homologous boosting for induction of either serum or bronchioalveolar anti-F1–V IgG1 responses, and (3) anti-F1 and anti-V total IgG responses were highest in animals primed intranasally and boosted by any route when compared to animals primed transcutaneously or subcutaneously. As with previously published studies, there were still significant levels of circulating anti-F1–V antibodies 1 year post-primary immunization. These studies provide important insights into the development of new-generation biodefense vaccines.

Keywords: Vaccination; Plague; *Y. pestis* F1–V

1. Introduction

The etiologic agent of plague is the Gram-negative bacterium *Yersinia pestis*. *Y. pestis* is a concern as one of the microorganisms with potential for use against civilian or military populations as an agent of biological warfare or biological terrorism. In that case, the pneumonic form of plague would be the most likely outcome. Pneumonic plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers.

A number of potential subunit vaccines against plague have been evaluated for immunogenicity and protective efficacy. The two most promising are the *Y. pestis* proteins F1 and V. F1 is a capsular protein located on the surface of the bacterium and the V-antigen is a component of the type-III secretion system. In previous studies, combined immunization with native F1 and recombinant V in a two-dose regimen afforded full protection of mice against subcutaneous (SC) challenge with *Y. pestis* [1] and the anti-F1 and -V titers, especially of the IgG1 sub-class, correlated significantly with protection in BALB/c mice. Male and female CBA, C57/BL6 and CB6F1 mice were also protected against SC and aerosol challenge with *Y. pestis* following immunization with two doses of F1 and V [2]. The combination or fusion of F1 and V has been shown to have an additive protective effect in the murine model when compared to either antigen alone [1,3-

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8]. In order to capture immunologic determinants of both molecules in a single antigen, Heath et al. [3] constructed a genetic hybrid of F1 and V, designated F1–V, consisting of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V-antigen. F1–V has been shown to provide protection against flea-borne SC and aerosol challenge, and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild type $F1^+$ *Y. pestis* or to naturally occurring $F1^-$ variants [3,9].

In the current study, we examine different prime–boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to further explore the ability of F1–V to promote the development of long-lasting, high-titer antibodies against this protein. We also examine the effect of different prime–boost regimes on the compartmentalization of the ensuing immune response. For parenteral immunization, F1–V was adsorbed to aluminum hydroxide, which is commonly used as an adjuvant for parenterally administered vaccines. Mucosally and transcutaneously administered proteins are usually not immunogenic and require the presence of an appropriate adjuvant. In the studies reported here, we utilize a mutant of the heat-labile enterotoxin of *Escherichia coli*, designated LT(R192G) that has been shown to be effective when administered mucosally (orally, rectally, and intranasally) or transcutaneously in a variety of animal models and in humans [10-28].

2. Materials and methods

2.1. Animal immunizations

Groups of 8–9-week-old female Swiss Webster mice (Charles River Laboratories) were immunized once (day 0) or twice (day 0 and day 28) with the recombinant F1–V fusion protein vaccine developed at the United States Army Medical Research Institute of Infectious Diseases [3]. LT(R192G) was prepared in our laboratory by galactose-affinity chromatography as previously described [29]. Mice were immunized SC, intranasally (IN), or transcutaneously (TC) and then boosted by the same (homologous) or a different (heterologous) route (Table 1). Mice immunized SC received 10 μg of recombinant F1–V adsorbed to 0.19 mg of an aluminum hydroxide adjuvant (2.0% Alhydrogel batch no. 3275; Superfos Biosector, Vedbaek, Denmark) in a final volume of 100 μl. Mice immunized IN received 5 μg of recombinant F1–V admixed with 5 μg LT(R192G) in a final volume of 9.6 μl in one nostril following brief exposure to Isofluorane. Mice previously anesthetized by intraperitoneal injection with Ketamine–Xylazine were immunized TC with 35 μg F1–V admixed with 25 μg LT(R192G) in a final volume of 50 μl applied to freshly shaved ventral skin. These doses were based upon preliminary studies in our laboratory and upon studies published elsewhere.

Table 1
Immunization schedule

Priming dose	Boosting dose at day 28		
Subcutaneous (SC)	SC	IN	TC
Intranasal (IN)	SC	IN	TC
Transcutaneous (TC)	SC	IN	TC

2.2. Measurement of serum and bronchioalveolar lavage antibody

Mice were sacrificed by CO_2 inhalation in groups of five from each of the three primary immunization groups on day 28, and from each of the nine homologous and heterologous prime–boost groups on days 59, 191, and 385. Blood was obtained from each animal by cardiac puncture. Lung lavage fluid was collected from each animal by exposing the trachea, making a small incision, inserting and securing an 18-gauge needle, and aspirating 1 ml of PBS three times before final withdrawal. Serum and bronchioalveolar lavage (BAL) fluid were examined for the presence of anti-F1–V, anti-F1 or anti-V antibodies by ELISA. Briefly, ELISA plates were coated with 0.1 μg per well of recombinant F1–V, F1 or V in 100 μl sodium carbonate buffer. Following overnight incubation at 4 °C, plates were washed in PBS containing 0.05% Tween 20, and two-fold serial dilutions of the serum or BAL from immunized animals were applied. After incubation for 1 h at room temperature, plates were washed and a 1:400 dilution of goat anti-mouse IgG or IgG1 labeled with alkaline-phosphatase was added and incubation continued for 1 h at room temperature. Plates were washed and the substrate PNPP was added. The reaction was stopped with 2N NaOH and the plates were read at an optical density at 405 nm. For quantitative analysis, concentrations of serum and BAL anti-F1–V, -F1, or -V IgG or IgG1 were determined by non-linear regression from a standard curve of mouse myeloma IgG1 (Sigma Chemical Co., Saint Louis, MO, USA) serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentration ± S.E.M.

2.3. Statistical analysis

Statistical analyses were performed using a one-way analysis of variance with the Bonferroni Multiple Comparison post-test.

3. Results

3.1. Serum and BAL anti-F1–V IgG1 response following homologous prime–boost

The purpose of this group of experiments was to compare three different routes of immunization (IN, TC, and SC) for the ability to induce high-titer anti-F1–V serum and BAL antibody responses following one or two immunizations with

Table 2
Serum anti-F1–V IgG1 (μg/ml) ± S.E.M.

	Day 28	Day 59	Day 191
IN (prime only)	5 ± 1	–	–
TC (prime only)	0	–	–
SC (prime only)	207 ± 82	–	–
IN × IN	–	519 ± 114	417 ± 103
IN × TC	–	461 ± 116	363 ± 131
IN × SC	–	1124 ± 268	1454 ± 358
TC × IN	–	97 ± 55	41 ± 6
TC × TC	–	53 ± 12	55 ± 41
TC × SC	–	412 ± 124	473 ± 187
SC × IN	–	702 ± 93	700 ± 226
SC × TC	–	868 ± 228	1678 ± 298
SC × SC	–	779 ± 132	859 ± 91

the priming dose and booster dose delivered by the same (homologous) route. Mice were immunized once or twice with F1–V adsorbed to alum (SC) or admixed with LT(R192G) (IN or TC), and groups of five animals from each regimen were sacrificed at days 28, 59, 191, and 385 post-primary immunization. Since previous studies by Williamson et al. [1] had shown that anti-F1 and -V IgG1 responses correlated significantly with protection, serum and BAL anti-F1–V IgG1 responses were determined by ELISA.

As shown in Fig. 1 and Tables 2 and 3, a single primary immunization with F1–V by any of the three routes induced no (TC) or only a minimal (IN or SC) anti-F1–V serum or BAL antibody response at 28 days post-primary immunization. The maximum serum (207 ± 82 μg/ml) and BAL (531 ± 163 ng/ml) anti-F1–V IgG1 response achieved following a single primary immunization was by SC delivery. There was no detectable serum or BAL anti-F1–V antibody response following a single TC immunization.

Following a single homologous boosting dose administered at day 28, serum and BAL anti-F1–V IgG1 responses increased in all three groups with peak responses observed by day 59. Specifically, anti-F1–V responses after day 59 were never significantly ($p > 0.05$) greater than the day 59 responses within any group. The highest responses were observed following either IN or SC immunization, which were not different from one another at day 59 ($p > 0.05$). With respect to concentrations of anti-F1–V antibodies, both IN and SC regime induced significantly higher levels of serum and BAL anti-F1–V IgG1 than did TC immunization with the same antigen ($p < 0.01$). To be clear, homologous TC prime–boost did induce significant levels of serum (53 ± 12 μg/ml) and BAL (297 ± 132 ng/ml) anti-F1–V IgG1 at day 59, just not to the same level as that obtained following either IN or SC homologous prime–boost.

Table 3
BAL anti-F1–V IgG1 (ng/ml) ± S.E.M.

	Day 28	Day 59	Day 191
IN (prime only)	23 ± 23	–	–
TC (prime only)	0	–	–
SC (prime only)	531 ± 163	–	–
IN × IN	–	4207 ± 1197	893 ± 267
IN × TC	–	7466 ± 3343	1245 ± 728
IN × SC	–	3109 ± 623	1020 ± 206
TC × IN	–	562 ± 196	177 ± 58
TC × TC	–	297 ± 132	50 ± 31
TC × SC	–	3104 ± 1687	471 ± 172
SC × IN	–	1973 ± 399	2211 ± 1038
SC × TC	–	9336 ± 3333	2374 ± 528
SC × SC	–	3145 ± 1050	1244 ± 190

With respect to duration of response (see Fig. 1), there was no significant decline in serum anti-F1–V IgG1 through 6 months post-primary immunization within groups of animals immunized either IN, TC or SC, while there was a significant decrease in BAL anti-F1–V IgG1 between 2 and 6 months. Specifically, the serum anti-F1–V response at day 191 was not significantly different from the serum anti-F1–V response at day 59 following either IN, TC or SC prime–boost ($p > 0.05$). In contrast, the BAL anti-F1–V response declined between 2 and 6 months post-primary immunization. When comparing IN and SC homologous prime–boost, SC immunization induced higher and more sustained levels of serum, but not BAL, anti-F1–V than did IN prime–boost. Thus, the serum anti-F1–V IgG1 response at 191 days post-primary immunization following SC prime–boost (859 ± 91 μg/ml) was significantly higher than that obtained following IN prime–boost at day 191 (417 ± 103 μg/ml) ($p < 0.01$).

With respect to distribution, both IN and SC prime–boost induced significant and sustained levels of both serum and BAL anti-F1–V IgG1. Taken together, these results demonstrate that IN and SC immunizations are both effective and essentially equivalent for induction of serum and BAL anti-F1–V IgG1 responses when a single booster dose is administered by the same (homologous) route. TC homologous prime–boost was less effective than either IN or SC homologous prime–boost.

3.2. Serum and BAL anti-F1–V IgG1 response following heterologous prime–boost

Heterologous boosting offers the possibility of increasing the magnitude or duration of the immune response when compared to homologous boosting and may also influence the compartmentalization of that response. For these studies, animals received a primary immunization by one route and then a single booster dose by the same or an alternate route (Table 1). As above, groups of five animals from each regimen were sacrificed at days 28 (no boost), 59, 191, and 385 post-primary immunization and examined for the presence of serum and BAL anti-F1–V IgG1 by ELISA.

As seen in Fig. 2A and Tables 2 and 3, animals primed IN and then boosted SC (circle) developed higher levels of serum anti-F1–V than did animals primed IN and boosted either IN (square), or TC (triangle) ($p < 0.05$). This IN-prime–SC-boost response remained elevated through 6 months post-primary immunization, and declined thereafter, such that by day 385 there were no significant differences between groups. The serum anti-F1–V responses in animals primed IN and boosted

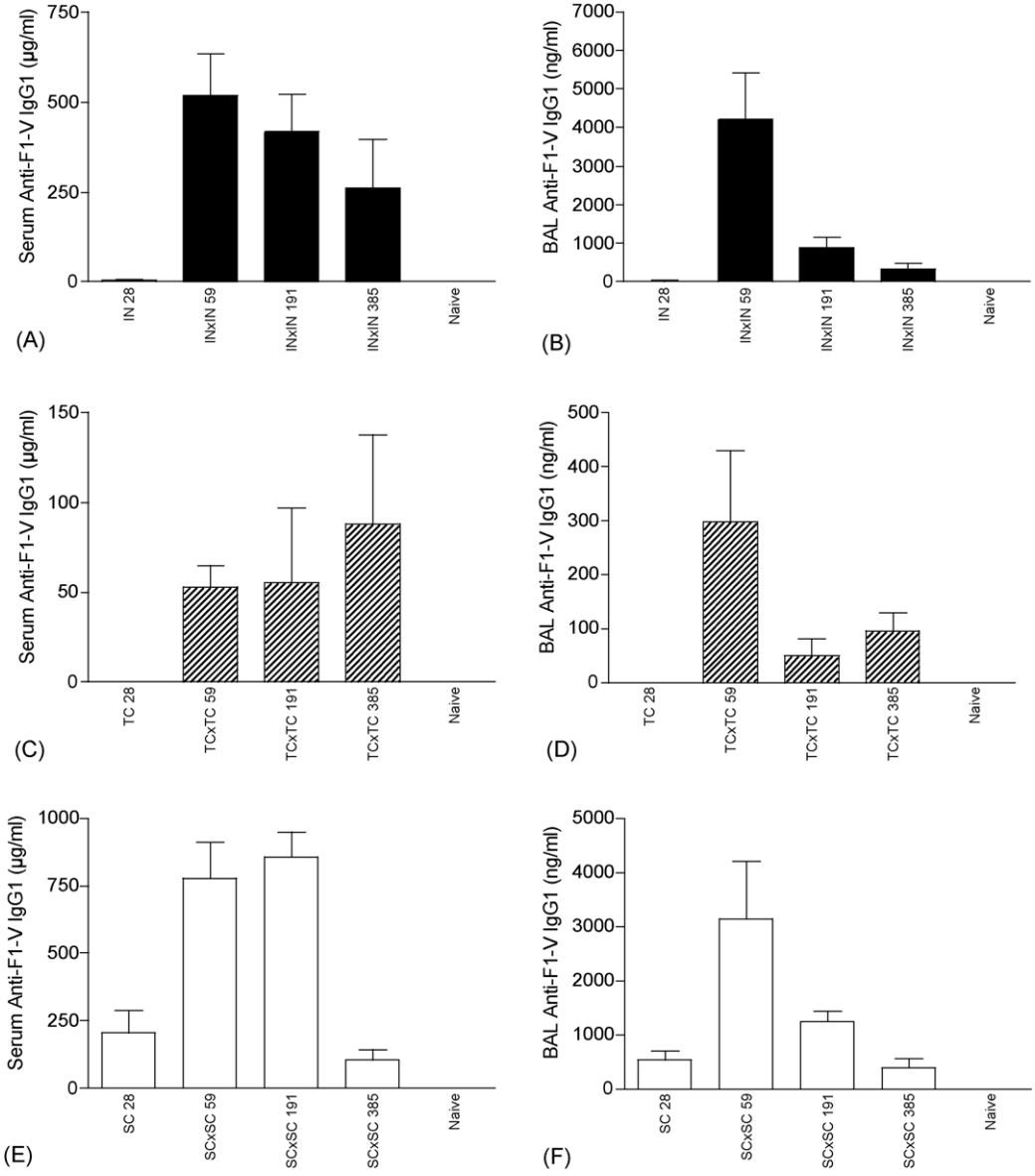

Fig. 1. Swiss/Webster mice were primed IN (panels A and B), TC (panels C and D), and SC (panels E and F) on day 0 and then boosted by the same (homologous) route on day 28. Five animals from each group were sacrificed on days 28 (no boost), 59, 191, and 385 following the primary immunization. Blood was collected by cardiac puncture (serum: panels A, C, and E) and lung washes were collected by bronchioalveolar lavage (BAL: panels B, D, and F). Concentrations of IgG1 were determined by ELISA by non-linear regression against an IgG1 standard curve.

IN (square) or TC (triangle) also remained elevated through 6 months, although clearly at a lower level than that obtained with IN priming and SC boosting (Fig. 2A). The BAL anti-F1–V IgG1 responses at day 59 for animals primed IN were highest in animals boosted TC ($p < 0.05$) when compared to animals boosted SC (Fig. 2B) while IN and SC boosting of IN primed animals produced responses that were indistinguishable from one another. All BAL anti-F1–V IgG1 responses dropped to baseline by 6 months post-primary immunization.

Transcutaneous immunization is an attractive needle-free alternative to parenteral immunization and numerous studies have demonstrated that transcutaneous homologous prime–boost can be effective for induction of antigen-specific serum and BAL antibody responses [30–34]. However, the effect of TC priming and heterologous boosting has not been widely studied. As shown in Fig. 2C and D, animals primed TC and then boosted SC developed higher levels of serum (Fig. 2C) and BAL (Fig. 2D) anti-F1–V than did animals primed TC and boosted either IN or TC ($p < 0.05$). However, the anti-F1–V levels achieved by TC priming and heterologous boosting (Fig. 2C and D) did not reach the levels achieved by IN priming and heterologous boosting (Fig. 2A and B). Animals primed TC and then boosted either TC or IN developed equivalent serum and BAL anti-F1–V responses.

Parenteral prime and boost is the more traditional route of vaccine delivery. In our studies, animals primed SC and

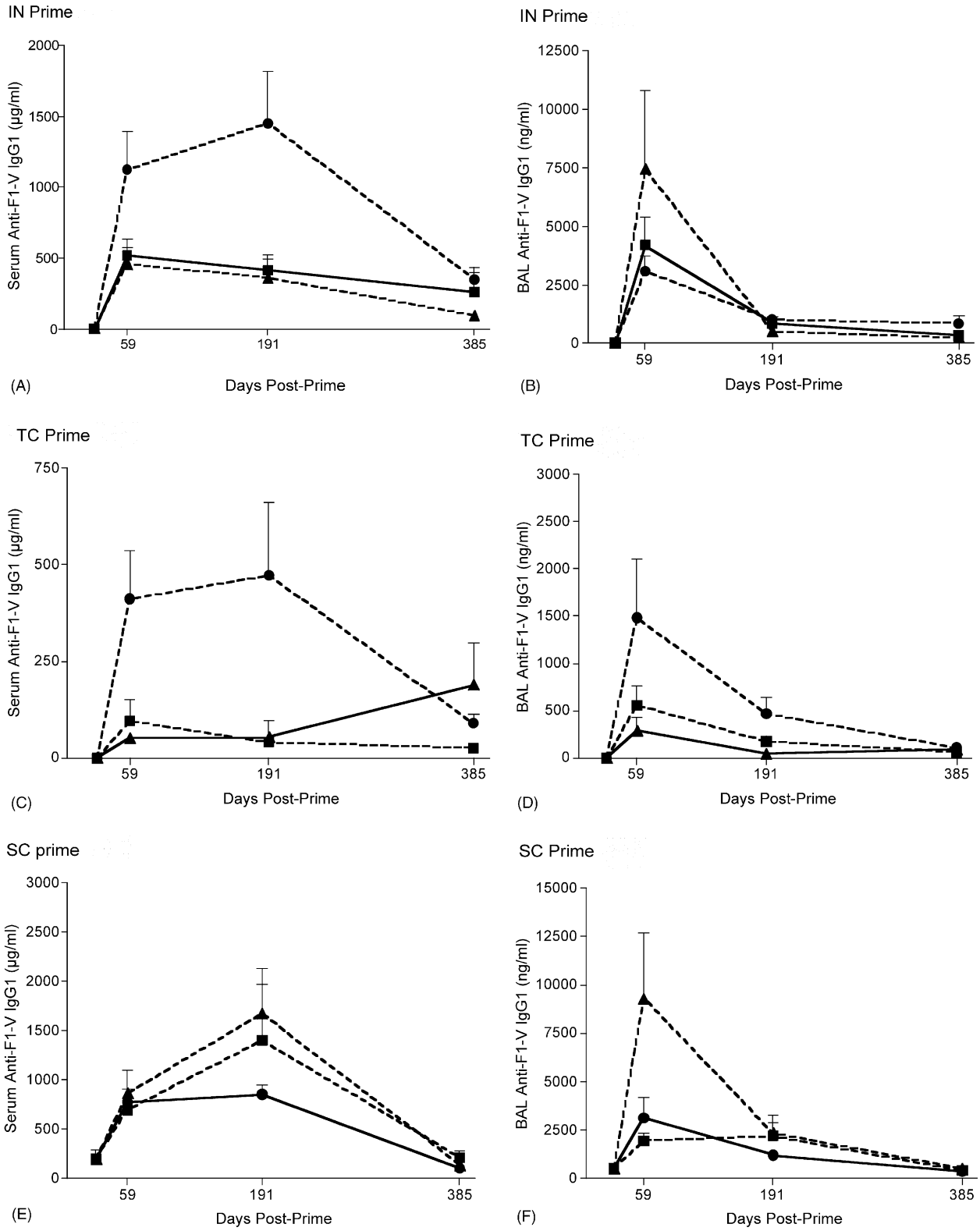

Fig. 2. Swiss/Webster mice were primed IN (panels A and B), TC (panels C and D), and SC (panels E and F) on day 0 and then boosted IN (closed rectangle), TC (closed triangle), or SC (closed circle) on day 28. Five animals from each group were sacrificed on days 28 (no boost), 59, 191, and 385 following the primary immunization. Blood was collected by cardiac puncture (serum: A, C, and E) and lung washes were collected by bronchioalveolar lavage (BAL: B, D, and F). Concentrations of IgG1 were determined by ELISA by non-linear regression against an IgG1 standard curve. Solid lines represent homologous boosting. Dotted lines represent heterologous boosting.

boosted IN or TC had higher levels of serum anti-F1–V IgG1 through 6 months post-primary immunization than animals primed SC and boosted SC ($p < 0.05$) (Fig. 2E). The highest level of BAL anti-F1–V following SC priming was obtained by TC boosting ($p < 0.05$) when compared to either IN or SC boosting (Fig. 2F). As with IN priming, the BAL responses were maximal at 2 months post-primary immunization and declined significantly by 6 months.

These findings demonstrate that the highest and most sustained levels of serum anti-F1–V IgG1were obtained following IN-priming and SC-boosting, or SC-priming and either IN- or TC-boosting, while the highest levels of BAL anti-F1–V IgG1were obtained following either IN or SC-priming and TC-boosting. These studies clearly demonstrate that heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1–V IgG1 responses. In no case was heterologous boosting inferior to homologous boosting.

3.3. F1- and V-specific IgG responses following heterologous prime–boost

In order to determine the effect of homologous and heterologous prime–boost on the serum response to both F1 and V, separate F1 and V ELISA assays were performed on sera from the two peak-time points for each group, days 59 and 191 post-primary immunization. For these studies, in contrast to the studies above where anti-F1–V IgG1 responses were determined, total IgG serum anti-F1 and anti-V responses were evaluated to obtain an assessment of the broader antibody response to each of the two antigens. As seen in Fig. 3, anti-F1 and -V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. This is an important observation since the correlate of protection is generally regarded to be serum anti-F1 or anti-V IgG1, and the higher magnitude of total IgG response, consisting of different isotypes of IgG, following IN immunization may not, in fact, correlate with protection. In general, serum anti-F1 and anti-V total IgG responses diminished over time, the notable exception being anti-V responses in animals that were primed SC and boosted heterologously (IN or TC), which may have implications for long-term protection.

4. Discussion

Plague is endemic in rodent populations in many regions of the world, including the U.S., and cases of bubonic plague have been reported in humans. More importantly, *Y. pestis* has the potential to be used as an agent of biological warfare or bioterrorism on military and civilian populations. For these reasons, designing a vaccine that protects against both forms of plague is a high priority. The previously approved killed whole-cell vaccine required multiple doses, had significant reactogenicity, did not provide protection against pneumonic

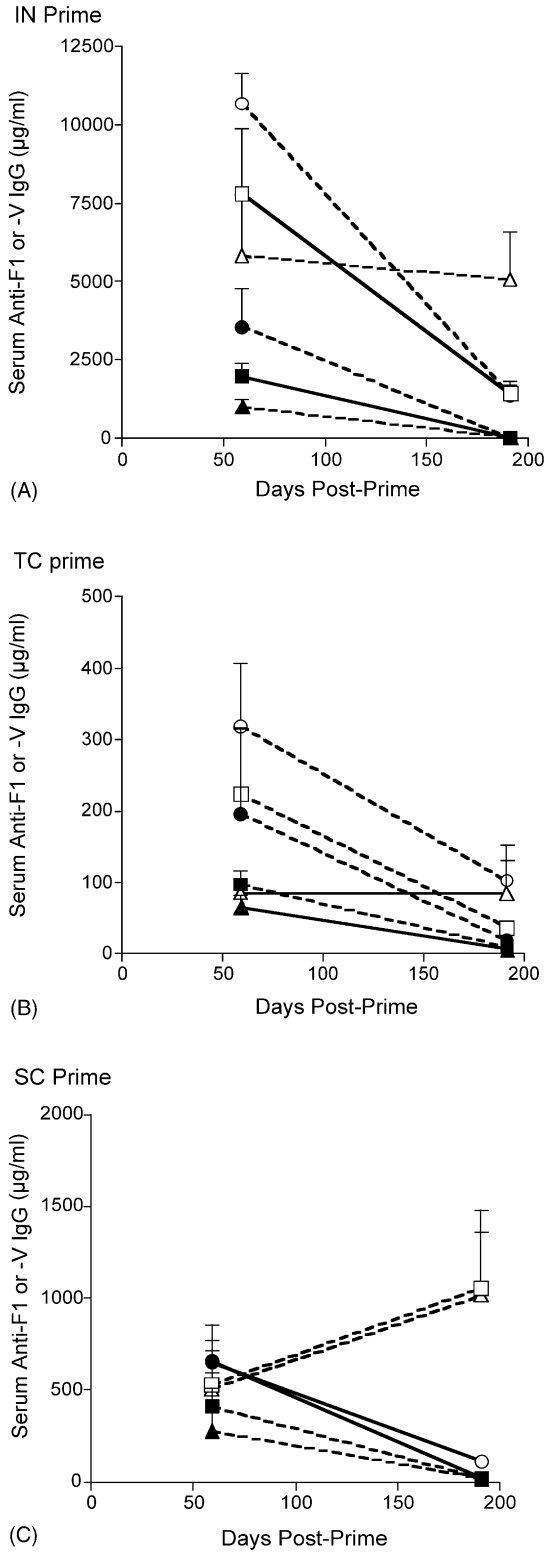

Fig. 3. Swiss/Webster mice were primed IN (panel A), TC (panel B), and SC (panel C) on day 0 and then boosted IN (rectangle), TC (triangle), or SC (circle) on day 28. Serum was analyzed from five animals in each group sacrificed on days 59 and 191 following the primary immunization. Concentrations of IgG were determined by ELISA by non-linear regression against an IgG standard curve. Open symbols are serum anti-V IgG. Closed symbols are serum anti-F1 IgG. Solid lines represent homologous boosting. Dotted lines represent heterologous boosting.

plague, and consequently, is no longer approved for use in the U.S. The EV76 live attenuated vaccine has been shown to cause disease in mice and produce side effects in monkeys and has never been approved for use in the U.S. [35–37]. Recent attempts to generate new vaccines against plague include the use of liposome formulated formalin-killed whole cells [38], new mutants of *Y. pestis* as live attenuated vaccines [39], oral immunization with attenuated mutants of *Salmonella enterica* serovar Typhimurium and *S. typhi* expressing *Y. pestis* antigens [40-42], DNA vaccines coding for F1 [43], and purified F1 or V or combinations of F1 and V [3,6,8,44,45].

Most studies to date with the F1–V fusion protein have involved parenteral immunization with F1–V administered with different adjuvants, including aluminum salts [1,6,46], incomplete Freund's adjuvant [8], and the Ribi adjuvant system [47]. All of these strategies lead to protection against challenge in the mouse model. Both serum IgG and serum IgG transudated into the lung are thought to contribute to protection against inhaled *Y. pestis* [6]. Indeed, even a single subcutaneous 30 μg dose of F1 + V or F1–V adsorbed to alum was sufficient to protect mice against aerosol challenge one year after immunization [46]. High titers of serum anti-F1 and anti-V IgG1 are clearly correlated with protection, at least in mice. There is no evidence to indicate that parenteral immunization will be effective in preventing plague in humans following aerosol exposure. *Y. pestis* causes a primary pneumonia in the lungs subsequent to aerosol exposure (in contrast to inhalational anthrax which starts as a systemic disease) and mice may not be the best model to demonstrate whether mucosal immunity will contribute to protection. Consequently, immunization strategies that induce both systemic and mucosal responses, especially at the level of the respiratory mucosa, may provide better protection than parenteral immunization in non-rodent species.

Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal, oral, and rectal) and transcutaneous immunization in the presence of an appropriate adjuvant has been shown to induce humoral and cellular immune responses in both the systemic and mucosal compartments of immunized animals. Alternating routes for delivery of the priming dose and booster dose in immunizations, so-called 'prime–boost' strategies, have also been examined for the ability to induce high-titer, long-lasting humoral responses and have the potential to direct or redirect the immune response to one compartment or another.

In the current study, we examined different prime–boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of recombinant F1–V to promote the development of long-lasting, high-titer antibodies. We also examined the effect of different prime–boost regimes on the compartmentalization of the ensuing immune response.

The most significant findings of the study reported here are that (1) IN and SC immunizations are both effective and essentially equivalent for induction of serum and BAL anti-F1–V IgG1 responses when a single booster dose is administered by the same (homologous) route, (2) heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1–V IgG1 responses, and (3) anti-F1 and -V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. As with previously published studies, there were still detectable levels of circulating anti-F1–V antibodies even 1 year post-primary immunization. It remains to be determined if the different immunization strategies are equally effective in protection against aerosol challenge, if there are qualitative differences in the responses or, more importantly, if solid protection can be achieved in species other than rodents (e.g., non-human primates) by altering the immunization route.

The observation that heterologous boosting may, in some cases, produce higher and more sustained antibody responses than homologous boosting is consistent with other reports that have examined this question [38,48–52]. Most recently, Lauterslager et al. [48] demonstrated that oral (PO) boosting with ovalbumin was more effective in animals primed IN, SC, or intraperitoneally than PO (homologous) boosting with the same antigen. Similarly, Nicholas et al. [50] demonstrated that SC immunization was effective for priming animals subsequently boosted SC or IN (but not PO) with chimeric virus particles expressing a 17-mer peptide sequence from canine parvovirus (CPMV). In those studies, animals primed IN and boosted SC developed significantly higher serum anti-CPMV IgG2a responses than did animals primed IN and boosted IN. In the study of Baca-Estrada et al. [38], mice immunized SC with formalin-killed whole cells and then boosted IN with formalin-killed whole cells, either alone or formulated in liposomes, developed higher serum and BAL anti-*Y. pestis* antibody and higher systemic cell-mediated immune responses than did animals boosted SC. Our findings are in agreement with these studies, all of which demonstrate that heterologous boosting can be as or more effective than homologous boosting for induction of serum antibodies. Since serum IgG1 has been shown to be protective against aerosolized *Y. pestis*, mucosal IgA was not examined in the current study. However, Baca-Estrada et al. [38], Lauterslager et al. [48], and Nicholas et al., [50] each demonstrated that heterologous boosting could also induce significant mucosal IgA responses.

The fact that IN and TC boosting of SC-primed animals generated higher levels of anti-F1–V antibodies than homologous SC-boosting is interesting and could be explained by the distribution of T effector–memory cells to the peripheral tissues following SC-priming where they would be available to interact with cognate antigen applied mucosally or transcutaneously in the context of an appropriate adjuvant (e.g. LT(R192G) [53]). However, SC boosting of IN- and TC-primed animals was also more effective than homologous IN- or TC-boosting for induction of serum anti-F1–V antibodies,

suggesting that a more global immunological phenomenon may be functioning here. Moreover, the adjuvant employed for IN and TC immunizations may also play a role. A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells as well as interacting directly with T-helper cells, B cells, and epithelial cells. Both Lauterslager et al. [48] and Nicholas et al. [50] utilized cholera toxin as a mucosal adjuvant. Clearly, the role of the adjuvant in controlling these outcomes requires further investigation.

The amount of antigen delivered by each route may also contribute to the outcome. Thus, the observation that both IN and SC regimen induced significantly higher levels of serum and BAL anti-F1–V IgG1 than did TC immunization with the same antigen (Fig. 2) may be influenced by the amount of antigen delivered. It is possible that the responses to TC prime and boost would be higher if larger amounts of antigen were applied or other methods were used to make uptake of transcutaneously administered antigens more efficient.

In and of itself, the observation that immunization by one route can prime for a secondary response by another route is important. In practical terms alone, especially in an imminent or post-release bioterrorism event, the ability to administer a parenteral priming dose and, at the same time, distribute a follow-on patch, pill, or nasal applicator that could be self-administered would greatly improve national preparedness.

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Protection against Aerosolized *Yersinia pestis* Challenge following Homologous and Heterologous Prime-Boost with Recombinant Plague Antigens

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A *Yersinia pestis*-derived fusion protein (F1-V) has shown great promise as a protective antigen against aerosol challenge with *Y. pestis* in murine studies. In the current study, we examined different prime-boost regimens with F1-V and demonstrate that (i) boosting by a route other than the route used for the priming dose (heterologous boosting) protects mice as well as homologous boosting against aerosol challenge with *Y. pestis*, (ii) parenteral immunization is not required to protect mice against aerosolized plague challenge, (iii) the route of immunization and choice of adjuvant influence the magnitude of the antibody response as well as the immunoglobulin G1 (IgG1)/IgG2a ratio, and (iv) inclusion of an appropriate adjuvant is critical for nonparenteral immunization.

Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal [i.n.], oral, and rectal) and transcutaneous (t.c.) immunization in the presence of an appropriate adjuvant have been shown to induce humoral and cellular immune responses in both the systemic and mucosal compartments of immunized animals. Alternating routes for delivery of the priming dose and booster dose in immunizations, so-called prime-boost strategies, have also been examined. Such prime-boost strategies could be particularly important in an imminent or postrelease bioterrorism event if it is possible to administer a parenteral priming dose and, at the same time, distribute a follow-up patch, pill, or nasal applicator that could be self administered. Such vaccine strategies would greatly improve national preparedness.

In a recent study, we evaluated different prime-boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of the recombinant *Yersinia pestis*-derived fusion protein (F1-V) to promote the development of long-lasting, high-titer antibodies (13). F1-V has been shown to provide protection against flea-borne, subcutaneous (s.c.), and aerosol challenge and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild-type $F1^+$ *Y. pestis* or to naturally occurring $F1^-$ variants (16, 17). The most significant finding of our previous study is that boosting by a different (heterologous) route than the priming dose can be as effective as or more effective than homologous boosting for induction of either serum or bronchoalveolar anti-F1-V immunoglobulin G1 (IgG1) responses.

In the current study, we examined the abilities of different prime-boost regimens with recombinant F1-V to protect mice against aerosol challenge with *Y. pestis*. We also examined the role of the coadministered adjuvant in inducing protection. For parenteral immunization, mice were immunized s.c. with 10 μg of F1-V alone or adsorbed to alum adjuvant (2.0% Alhydrogel, batch no. 3275; Superfos Biosector, Vedbaek, Denmark) brought to a final volume of 100 μl with 0.86 M NaCl. Mucosally and transcutaneously administered proteins are usually not immunogenic and also require the presence of an appropriate adjuvant. In the studies reported here, we utilized a mutant of the heat-labile enterotoxin of *Escherichia coli*, designated LT(R192G), that has been shown to be effective when administered mucosally (orally, rectally, or intranasally) or transcutaneously in a variety of animal models and in humans (2, 3, 5–7, 10, 12, 14, 19–24, 27–30, 32). Mice immunized i.n. received 5 μg of recombinant F1-V alone or admixed with 5 μg LT(R192G), brought to a final volume of 9.6 μl with TEAN (0.2 M NaCl, 0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3, pH 7.5), in one nostril following brief exposure to Isofluorane. Mice immunized t.c. received 35 μg F1-V alone or admixed with 25 μg LT(R192G), brought to a final volume of 50 μl with TEAN, applied to freshly shaved ventral skin following intraperitoneal injection of ketamine-xylazine. LT(R192G) was prepared in our laboratory by galactose-affinity chromatography as previously described (4). The vaccine antigen was a non-His-tagged version of the F1-V fusion protein, expressed by T7 polymerase with lactose operator control in *E. coli* strain BLR(DE3)/pPW731 and isolated to 99% purity with a four-column process (B.S. Powell, unpublished observation). Briefly, protein in clarified supernatant from disintegrated cells

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TABLE 1. Immunization groups

Immunization groups[a]	Prime antigen	Prime adjuvant	Boost antigen	Boost adjuvant
Naïve				
i.n. × i.n.	5 μg F1-V		5 μg F1-V	
INr × INr	5 μg F1-V	5 μg LT(R192G)	5 μg F1-V	5 μg LT(R192G)
i.n. × s.c.	5 μg F1-V		10 μg F1-V	
INr × SCa	5 μg F1-V	5 μg LT(R192G)	10 μg F1-V	10 μl Alum
s.c. × s.c.	10 μg F1-V		10 μg F1-V	
SCa × SCa	10 μg F1-V	10 μl Alum	10 μg F1-V	10 μl Alum
s.c. × t.c.	10 μg F1-V		35 μg F1-V	
SCa × TCr	10 μg F1-V	10 μl Alum	35 μg F1-V	25 μg LT(R192G)
t.c. × s.c.	35 μg F1-V		10 μg F1-V	
TCr × SCa	35 μg F1-V	25 μg LT(R192G)	10 μg F1-V	10 μl Alum

[a] For parenteral immunization, F1-V was administered alone (s.c.) or adsorbed to alum (SCa). For mucosal and transcutaneous immunizations, F1-V was administered alone (i.n. or t.c.) or admixed with the mucosal adjuvant LT(R192G) (INr or TCr).

was denatured with 6 M urea at room temperature. F1-V protein was then captured and refolded by anion exchange chromatography, further purified and concentrated over tandem hydrophobic interaction chromatography columns, and exchanged into phosphate-buffered saline by size exclusion chromatography before flash freezing and storage at −80°C. Protein identity, quality, and structure were measured by several methods and determined to be as predicted. Bioburden in the form of nucleic acid and endotoxin ranged from 3 to 13 ng/mg and 25 to 379 endotoxin units/mg, respectively.

Survival of immunized mice following aerosol challenge with *Y. pestis*. As shown in Table 1, groups of 8- to 9-week-old female Swiss Webster mice were immunized twice (day 0 and day 28) with F1-V alone (s.c., i.n., or t.c) or adsorbed to alum (SCa) or admixed with LT(R192G) (INr or TCr), and groups of 10 animals from each regimen were challenged by aerosol with 70 50% lethal doses of *Y. pestis* (CO92) on day 87 following the primary immunizing dose of F1-V. The mice were challenged using a dynamic 30-liter humidity-controlled Plexiglas whole-body exposure chamber. Total flow through the chamber was 19.5 liters/minute and was maintained at atmospheric pressure throughout the exposure. The test atmosphere was continuously sampled by use of a 6-liter-per-minute all-glass impinger (Ace Glass, Vineland, NJ). Heart infusion broth with 0.001% (vol/wt) Antifoam A (Sigma, St. Louis, MO) was used as impingement collection medium. Nebulizer and all-glass impinger samples were plated after the exposure to establish the aerosol concentration within the exposure chamber. By use of the exposure concentration, an inhaled dose was estimated by multiplying the empirically determined aerosol exposure concentration (CFU/liter air) in the chamber by the amount of air that was estimated to have been breathed by the mouse during the exposure. The cumulative air breathed by each mouse during the exposures was calculated by estimating the respiratory minute volume based on Guyton's formula as previously described (15). For this study, the average challenge dose over four runs of the aerosol system, expressed in total inhaled CFU/mouse was 1.5×10^6 CFU. Survival was monitored for 216 h. Differences in survival between groups challenged with *Y. pestis* CO92 were analyzed by the Kaplan-Meier method with the log-rank Mantel-Haenszel test. Differences with *P* values of 0.05 or less were considered significant.

As seen in Fig. 1 and Table 2, all animals in the naïve control group succumbed to infection following aerosol challenge with *Y. pestis* with a median survival time (MST) of 72 h. By contrast, 9/10 positive-control animals immunized with an SCa prime and an SCa boost (SCa × SCa) with F1-V adsorbed to alum survived for the 216-h postchallenge observation period ($P < 0.0001$). Equivalent protection (9/10) was observed in animals primed INr and boosted INr in the presence of the adjuvant LT(R192G). Thus, homologous prime and boost with F1-V by either of the two routes in the presence of an appropriate adjuvant can provide significant protection against aerosol challenge. This is an important finding because it demonstrates that homologous mucosal immunization in the presence of an appropriate adjuvant can induce protection equivalent to parenteral immunization.

A primary objective of the experiments reported here was to determine if heterologous boosting could provide equivalent protection against aerosol challenge compared to homologous boosting. As shown in Fig. 1 and Table 2, there were no differences in the survival rates of groups of animals primed INr and boosted SCa (10/10), primed SCa and boosted TCr (9/10), or primed TCr and boosted SCa (10/10) (heterologous prime-boost) compared to animals primed SCa and boosted SCa (9/10) or primed INr and boosted INr (9/10) (homologous prime-boost) if an appropriate adjuvant was included in the immunization. Differences in survival were observed if animals were immunized with F1-V without an adjuvant, depending upon the route of immunization. Thus, animals primed s.c. and boosted either s.c. or t.c. without adjuvant in either the priming or booster dose had equivalent protection (s.c. × s.c. = 7/10; s.c. × t.c. = 8/10) that was not significantly different from the levels of protection observed by any combination of routes that included adjuvant. By contrast, animals that were primed nonparenterally (e.g., i.n. or t.c.) with F1-V without adjuvant and then boosted i.n. or s.c. without adjuvant had significantly lower survival rates (i.n. × i.n. = 0/10; t.c. × s.c. = 4/10; i.n. × s.c. = 3/10) compared to animals primed and boosted with F1-V in the presence of the appropriate adjuvant. As shown in Fig. 1 and Table 2, none of animals primed i.n. and boosted i.n. without adjuvant survived beyond 144 h postexposure (MST = 96 h), compared to 9/10 animals that survived for the duration of the experiment when primed INr and boosted INr with F1-V

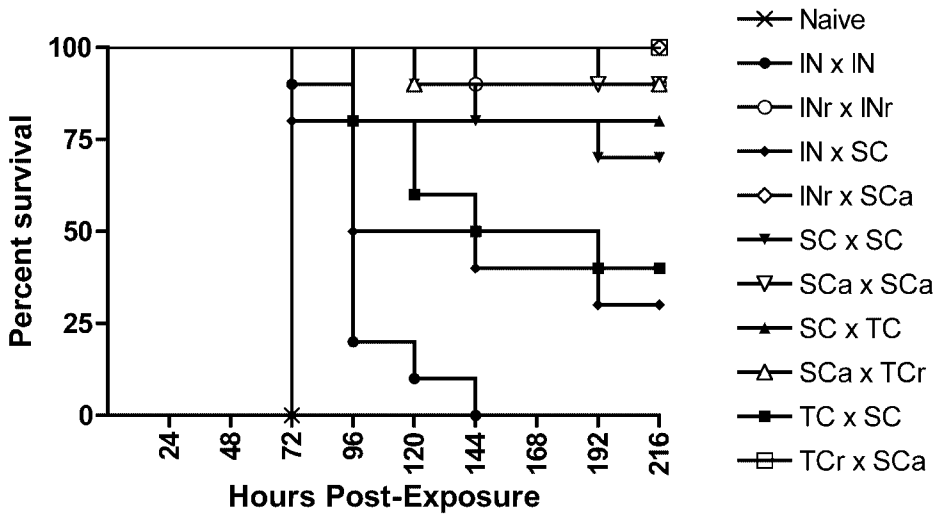

FIG. 1. Kaplan-Meier survival analysis of F1-V-immunized Swiss Webster mice after aerosol challenge with 70 50% lethal doses of *Y. pestis* (CO92) on day 87 postprimary immunization. There were no differences in survival rates of groups of animals primed INr and boosted SCa (10/10), primed SCa and boosted TCr (9/10), or primed TCr and boosted SCa (10/10) (heterologous prime-boost) compared to animals primed SCa and boosted SCa (9/10) or primed INr and boosted INr (9/10) (homologous prime-boost) if an appropriate adjuvant was included in the immunization. There were 10 mice per group.

admixed with the mucosal adjuvant LT(R192G) ($P < 0.0001$). Similarly, only 3/10 animals primed i.n. and boosted s.c. without adjuvant survived for the duration of the experiment (MST = 120 h) compared to 10/10 animals primed INr and boosted SCa with F1-V in the presence of adjuvant ($P = 0.0012$). Likewise, 4/10 animals primed t.c. and boosted s.c. without adjuvant survived for the duration of the experiment (MST = 168 h) compared to 10/10 animals primed TCr and boosted SCa with F1-V in the presence of adjuvant ($P = 0.004$).

Serum and bronchoalveolar lavage (BAL) anti-F1-V responses at the time of aerosol challenge following homologous or heterologous prime–boost. A cohort of mice immunized with F1-V adsorbed to alum (SCa) or admixed with LT(R192G) (INr or TCr) was sacrificed by CO_2 inhalation on the day corresponding to challenge (day 87 postprimary immunization) and their serum and BAL were examined for the presence of anti-F1-V, anti-F1, or anti-V antibodies by enzyme-linked immunosorbent assay (ELISA) on plates that were coated with 0.1 μg per well of recombinant F1-V, F1, or V in 100 μl bicarbonate buffer. Following overnight incubation at 4°C, plates were washed with phosphate-buffered saline containing 0.05% Tween 20, and twofold serial dilutions of the serum from immunized animals were applied. After incubation for 1 h at room temperature, plates were washed and a 1:400 dilution of goat anti-mouse IgG, IgG1, or IgG2a labeled with alkaline-phosphatase was added and incubation continued for 1 h at room temperature. Plates were washed, and the substrate paranitrophenyl phosphate was added. For quantitative analysis, concentrations of serum anti-F1-V, anti-F1, or anti-V IgG, IgG1, or IgG2a were determined by nonlinear regression from a standard curve of mouse myeloma IgG1 or IgG2a (Sigma Chemical Co., St. Louis, MO) serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentrations ± standard errors of the means (SEM). Statistical analyses were performed by using a one-way analysis of variance with Bonferroni's multiple comparison posttest. Statistical comparisons were performed with Prism version 4.0 (GraphPad Software Inc., San Diego, Calif.).

TABLE 2. Survival of immunized mice following *Y. pestis* aerosol challenge

Immunization groups[a]	% Survivors (216 h)	Median survival time (h)[b]
Naïve	0	72
i.n. × i.n.	0	96
INr × INr	90	N/A
i.n. × s.c.	30	120
INr × SCa	100	N/A
s.c. × s.c.	70	N/A
SCa × SCa	90	N/A
s.c. × t.c.	80	N/A
SCa × TCr	90	N/A
t.c. × s.c.	40	168
TCr × SCa	100	N/A

[a] For parenteral immunization, F1-V was administered alone (s.c.) or adsorbed to aluminum hydroxide (SCa). For mucosal and transcutaneous immunizations, F1-V was administered alone (i.n. or t.c.) or admixed with the mucosal adjuvant LT(R192G) (INr or TCr).

[b] Median survival time is the time at which 50% of the subjects have died. This value is not applicable (N/A) for groups with >50% survival.

Serum anti-F1-V IgG, IgG1, and IgG2a, as well as the serum anti-F1 and anti-V IgG responses in animals immunized with F1-V in the presence of an appropriate adjuvant, are shown in Table 3 and Fig. 2. Consistent with our previous findings, heterologous boosting was as effective as, or more effective than, homologous boosting for induction of significant anti-F1-V responses in immunized animals. The highest concentration of serum anti-F1-V IgG was obtained by heterologous prime-boost [INr prime with F1-V admixed with LT(R192G) and SCa boost with F1-V adsorbed to alum], and that was also reflected in the concentrations of anti-F1-V IgG1 and IgG2a (Table 3). With respect to serum anti-F1-V IgG1 and IgG2a

TABLE 3. Serum anti-F1-V (mean μg/ml ± standard error of the mean)

Immunization groups[a]	IgG	IgG1	IgG2a	IgG1/IgG2a ratio
Naïve	0	0	0	0
INr × INr	68 ± 27	14 ± 5	48 ± 31	0.3
INr × SCa	2,524 ± 1,427	443 ± 239	746 ± 495	0.6
SCa × SCa	374 ± 170	300 ± 201	53 ± 39	5.7
SCa × TCr	91 ± 40	46 ± 34	14 ± 5	3.3
TCr × SCa	58 ± 7	22 ± 4	9 ± 2	2.4

[a] Swiss Webster mice were primed INr, TCr, or SCa on day 0 and then boosted by the same route (homologous) or a different route (heterologous) on day 28. Animals were sacrificed on day 87 following the primary immunization. Blood was collected by cardiac puncture and analyzed by ELISA. The results obtained are expressed as the mean concentrations ± SEM. There were five mice per group.

ratios, animals that were primed INr had relatively lower IgG1/IgG2a ratios (INr × INr = 0.3; INr × SCa = 0.6) than did animals that were primed SCa or TCr (SCa × SCa = 5.7; SCa × TCr = 3.3; TCr × SCa = 2.4), with the most pronounced IgG1/IgG2a ratio resulting from SCa priming and SCa boosting with F1-V adsorbed to alum (Table 3). This shift in IgG1/IgG2a ratio could have resulted from either a route of immunization or adjuvant effect. With respect to BAL, all immunization groups that included adjuvant, regardless of route, developed significant levels of anti-F1-V IgG and IgG1. Animals that were primed INr and boosted SCa had the highest levels of overall BAL anti-F1-V IgG and anti-F1-V IgG1, and only those animals had detectable levels of BAL anti-F1-V IgG2a (data not shown). Additionally, BAL anti-F1-V IgA was not detected, and the concentration of BAL anti-F1-V IgG roughly corresponded to the level of serum anti-F1-V IgG, most likely indicating transudation of serum IgG into the BAL and not an active secretory process. Alternatively, the level of anti-F1-V BAL IgA may have been below the level of detection or may have peaked at a time point different than the sample time points in the experiments reported here. Serum anti-F1 IgG and anti-V IgG responses are shown in Fig. 2. Again, the highest concentration of either anti-F1 or anti-V was obtained by heterologous prime-boost [INr prime with F1-V admixed with LT(R192G) and SCa boost with F1-V adsorbed to alum]. Interestingly, there were no differences in protection against aerosol challenge between these immunization groups (Fig. 1 and Table 2).

The most significant findings of the study reported here are that (i) heterologous boosting protects mice as well as homologous boosting against aerosol challenge with *Y. pestis*, (ii) parenteral immunization is not required to protect mice against aerosolized plague challenge (i.n. × i.n. and s.c. × s.c. provide equivalent protection if an appropriate adjuvant is included in the vaccine formulation), (iii) the route of immunization and choice of adjuvant influence the magnitude of the antibody response as well as the IgG1/IgG2a ratio, and (iv) inclusion of an appropriate adjuvant is more critical for nonparenteral immunization.

The finding that a vaccine delivered by heterologous prime-boost can provide protection against aerosol challenge might have been predicted from our previous studies showing that the highest levels of anti-F1-V IgG1 were obtained by heterologous prime-boost. Related findings were reported by Eyles et al. (9), who demonstrated that t.c. application of F1 and V admixed with cholera toxin was effective for priming responses that could be boosted i.n. or intradermally and that t.c. application of F1 and V admixed with cholera toxin could effectively boost animals primed intradermally or i.n. However, the current study also demonstrates that i.n. priming in the context of an ADP-ribosylating adjuvant significantly lowers the serum IgG1/IgG2a ratio, indicating the development of more of a type 1 or mixed T-helper-cell response.

Moreover, INr × INr homologous prime-boost and SCa × TCr and TCr × SCa heterologous prime-boost all induced significantly lower levels of IgG1 than either SCa × SCa or INr × SCa immunization. Importantly, all of these groups had identical levels of protection against aerosol challenge. There are two possible explanations for the observed equivalent protection in the face of vastly different amounts of IgG1. First, there may be a threshold level of anti-F1 or anti-V IgG1 that is sufficient for protection and any of the combinations of routes in the context of an appropriate adjuvant can achieve that level. In that case, achieving the higher levels of antibody

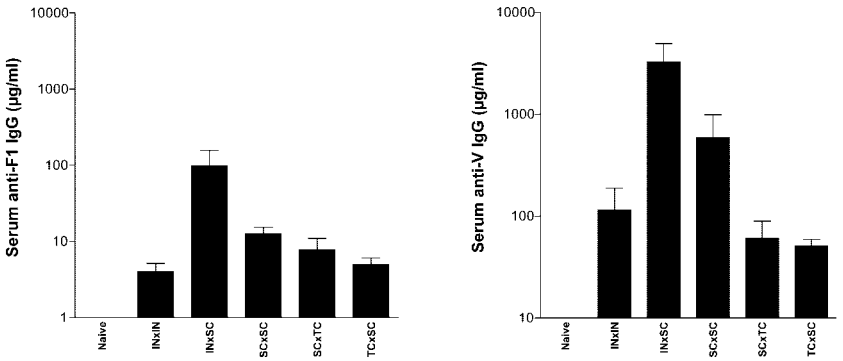

FIG. 2. Swiss Webster mice were primed INr, TCr, or SCa on day 0 and then boosted by the same route (homologous) or a different route (heterologous) on day 28. Animals were sacrificed on day 87 following the primary immunization. Blood was collected by cardiac puncture and analyzed by ELISA. Concentrations of serum anti-F1 or anti-V IgG were determined by nonlinear regression from a standard curve of mouse myeloma IgG1 serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentrations ± SEM. There were five mice per group.

would be important only if there was a concomitant increase in duration of circulating antibody or a relative increase in the challenge dose. The second possibility is that while anti-F1 or anti-V IgG1 may be correlated with protection, it may not be the sole protective factor. Indeed, a recent study by Elvin and Williamson (8) examined Stat6$^{-/-}$ and Stat4$^{-/-}$ mice to determine the relative importance of type 1 and type 2 immune responses in protection against plague challenge. Surprisingly, serum antibody responses to vaccination in both knockout strains were not different from wild-type controls with respect to levels of IgG or isotype profile. Moreover, Stat6$^{-/-}$ mice (unable to utilize type 2 cytokines interleukin 4 [IL-4] and IL-13) were highly protected against s.c. challenge, while Stat4$^{-/-}$ mice (inactivated IL-12 and interferon-γ-mediated immune mechanisms) were poorly protected, indicating that a type 1 immune mechanism, activated following Stat4 phosphorylation, may be essential for protection against plague. Thus, the undiminished protection following the observed shift to a type 1 or more mixed T-helper-cell response following i.n. priming in our study may reflect the contributions of both type 1 and type 2 responses to protection against aerosol challenge.

A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells, as well as interacting directly with T-helper cells, B cells, and epithelial cells (1, 11, 18, 25, 26, 31). We did not include antigen-only (nonadjuvant) controls in the cohort immunization study, but future studies comparing adjuvanted and nonadjuvanted immunization groups could resolve whether the IgG1/IgG2a ratio shift is a function of the route of immunization or adjuvant.

The discovery that immunization by one route can prime for a secondary response by another route and protect animals against high-dose lethal aerosol challenge has far-reaching implications, especially for national preparedness in a biodefense or emerging infectious disease crisis.

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